Design of immunogens that present the crown of the HIV-1 V3 loop in a conformation competent to generate 447-52D-like antibodies

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gp120 is a subunit of the envelope glycoprotein of HIV-1. The third variable loop region of gp120 (V3 loop) contains multiple immunodominant epitopes and is also functionally important for deciding cell-tropism of the virus. 447-52D is a monoclonal antibody that recognizes the conserved tip of the V3 loop in a β -turn conformation. This antibody has previously been shown to neutralize diverse strains of the virus. In an attempt to generate an immunogen competent to generate 447-52D-like antibodies, the known epitope of 447-52D was inserted at three different surface loop locations in the small, stable protein *Escherichia coli* Trx (thioredoxin). At one of the three locations (between residues 74 and 75), the insertion was tolerated, the resulting protein was stable and soluble, and bound 447-52D with an affinity similar to that of intact gp120. Upon immunization, the V3 peptide-inserted

Trx scaffold was able to generate anti-V3 antibodies that could compete out 447-52D binding to gp120. Epitope mapping studies demonstrated that these anti-V3 antibodies recognized the same epitope as 447-52D. Although the 447-52D-type antibodies were estimated to be present at concentrations of 50–400 μ g/ml of serum, these were not able to effect neutralization of strains like JRFL and BAL but could neutralize the sensitive MN strain. The data suggest that because of the low accessibility of the V3 loop on primary isolates such as JRFL, it will be difficult to elicit a V3-specific, 447-52D-like antibody response to effectively neutralize such isolates.

Key words: HIV-1, immunogen design, neutralizing antibody, stability, thioredoxin, V3 loop.

INTRODUCTION

It is well known that a significant fraction of strain-specific virus-neutralizing antibodies in the serum of HIV-1-infected individuals recognize the third hypervariable loop (V3) domain of the surface subunit of the envelope glycoprotein (gp120) of HIV-1 [2,3]. This epitope is also known to be the principal neutralizing domain of TCLA (T-cell line adapted) strains of HIV-1 [4–6]. There have been studies that highlight the potential importance of using the V3 loop as a target in vaccine development. In one of these studies, it was shown that passive administration of chimpanzees with murine monoclonal antibody against the V3 loop could protect them from challenge with TCLA strains of HIV-1 [7]. There has also been considerable debate regarding the accessibility of the V3 loop on primary isolates of the virus. Certain reports suggest that the V3 loops on gp120 isolated from patients can be relatively inaccessible [8–10], while other studies suggest that this region of the glycoprotein is accessible in primary isolates and can serve as a neutralization epitope [11–13]. Studies in which V3 loop peptides were used as immunogens showed that these sequences could elicit antibodies that were type-specific and displayed little, if any, cross-reactivity [4,14]. There have also been studies where V3-specific, neutralizing mAbs (monoclonal antibodies) were derived from cells of HIV-1-infected individuals [15]. One study also reports that C-terminal fusion of the V3 loop to the N-terminal domain of the murine leukaemia virus surface protein, gp70, is a better selecting antigen to isolate cross-reactive neutralizing antibodies than linear V3 loop peptides [11].

One useful characteristic of the V3 epitope is the ease with which it can be mimicked with a synthetic peptide. Antibodies able to neutralize TCLA strains are produced upon immunization with these linear peptides [7]. There have also been other attempts to use V3 as an effective antigen. In one approach, tandem copies of V3 loops derived from various strains of HIV-1 were fused together at the gene level to produce a multi-strain V3 loop antigen [16]. In another approach, cyclic peptides that attempted to mimic the probable V3 conformation in the virus have also been used for immunization [17–20].

In spite of the extensive work that has been done on the V3 loop, it still remains unknown whether the V3 loop in an appropriate native conformation can elicit anti-V3 broadly cross-reactive neutralizing antibodies. There can be two approaches taken to answer the question. Firstly, the antibody response against gp120 can be immunofocused on the V3 loop by antigenic masking of the other immunodominant regions of gp120 [21,22]. However, such an approach is technically difficult. The second possible solution is the design of a V3 loop construct that binds a neutralizing antibody with an affinity similar to gp120. This can subsequently be used as an immunogen in efforts to elicit broadly cross-reactive neutralizing anti-V3 antibodies. In the present study, we have followed the second approach to generate a V3 loop derivative. NMR studies on free V3 loop peptides report the presence of a relatively unstructured ensemble of V3 molecules in solution [17,23–26]. NMR and crystal structures have been solved for V3 peptides in complex with a number of neutralizing antibodies [27–30], including mAb 447-52D [31]. Very recently [32], the

Abbreviations used: HRV, human rhino virus; mAb, monoclonal antibody; NHisTrx, *Escherichia coli* thioredoxin with an N-terminal hexahistidine tag; 33NHisTrxV3, NHisTrx with residues 305–320 of JRFL HIV-1 gp120 inserted between residues 33 and 34; 74NHisTrxV3(307), same as 74NHisTrxV3 but with additional mutations I307C/Y318C; 74NHisTrxV3(308), same as 74NHisTrxV3 but with additional mutations H308C/F317C; 74NHisTrxV3, same as 33NHisTrxV3 but with insertion between residues 74 and 75; 83NHisTrxV3, same as 33NHisTrxV3 but with insertion between residues 83 and 84; Ni-NTA, Ni²⁺-nitrilotriacetate; RU, response units; SPR, surface plasmon resonance; TCLA, T-cell line adapted; Trx, thioredoxin.

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structure of a V3-containing gp120 core has also been determined. In this structure, the V3 loop is extended and accessible. The tip of the V3 loop containing the conserved GPG amino acid sequence has a similar conformation to that seen previously in antibody peptide complexes [31,33] and in free peptide [34]. mAb 447-52D is a conformation-specific antibody directed towards the conserved tip of the V3 loop. This is able to neutralize a wider range of primary isolates than many other V3-directed antibodies [1,11]. There has been one report in which the V3 loop was inserted into a loop region of the coat protein of HRV14 (human rhinovirus type 14) [35]. The structure of the V3 peptide in this fusion protein was determined and found to interact with the HRV coat protein. Neutralization and immunization studies showed that this construct was able to bind to various neutralizing antibodies and also generate a weak neutralizing response against MN and TCLA strains of HIV-1. Although this study reported that the inserted peptide was able to bind to V3-specific neutralizing antibodies, binding affinities were not reported. In the present study, we report the design of V3 loop derivatives that attempt to mimic the conformation of the loop as it occurs in its 447-52D-bound form. The V3 loop peptide adopts a β -hairpin structure in this complex [31]. Although the entire V3 loop shows extensive sequence variation, the sequence of the tip of the β -hairpin is well conserved (GPGR in subtype B and GPGQ in subtypes A, C and D). There are various ways a β -hairpin can be designed while keeping the V3 sequence unchanged. In one approach, the β -hairpin can be formed by the design of disulfide-linked cyclic peptides. The problem with this approach lies in the fact that low mass peptides typically require covalent conjugation to a protein carrier in order to enhance immunogenicity and the chemical cross-linking step can modify the sequence and/or conformation of the peptide. It is also well known that disulfides introduced to cross-link the two strands in a β -hairpin introduce strain that induces subtle changes in the main chain geometry [36]. Such an approach had been used earlier to make random peptide libraries inserted in the active site loop of *Escherichia coli* Trx (thioredoxin) [37,38]. In the present study, we design V3 loop inserts in Trx following previously established strategies for peptide insertion in this protein and we report the discovery of an alternative and optimized site for insertion. These constructs were used to immunize guinea-pigs and characterize the immune response against the constrained V3 loop peptide in the context of a Trx scaffold. Trx was chosen as the scaffold because it is a small rigid, stable protein that is structurally well characterized and can be expressed to high levels in *E. coli*.

MATERIALS AND METHODS

Construct descriptions

gp120 refers to the JRFL isolate of gp120 (residues 1–505). Trx refers to *E. coli* Trx (residues 1–108). NHisTrx is a construct containing a (His)₆ tag at the N-terminus of Trx expressed under the control of the P_{BAD} promoter in plasmid pBADNHisTrx, which is a derivative of pBAD24 [39].

Construction of 33NHisTrxV3, 74NHisTrxV3, 83NHisTrxV3 and disulfide-containing derivatives

Plasmids encoding the insertion of a portion of the V3 loop of gp120 (amino acids 305–320) between residues 33 and 34, between residues 74 and 75 and between residues 83 and 84 of Trx (hereafter referred to as 33NHisTrxV3, 74NHisTrxV3 and 83NHisTrxV3 respectively or TrxV3 derivatives, collectively) were constructed as described below. The gene encoding

the V3 loop of gp120 was PCR-amplified from the vector V1jnstpaoptgp140 [40]. The gene was amplified with a 5' and 3' overhang of Trx sequence. For insertion of the loop between residues 33 and 34 of Trx, the V3 gene contained a 5' overhang with the sequence of Trx upstream of residue 33. The 3' overhang contained the sequence of Trx downstream of residue 34. The gene encoding Trx from residue 1 to 33 was PCR-amplified with a 3' overhang of V3 loop sequence. The gene encoding Trx from residue 34 to the end was PCR-amplified with a 5' overhang of V3 loop sequence. The three PCR products were mixed and subjected to overlap PCR. The resulting products were digested with NdeI and HindIII and cloned into pBADNHisTrx digested with the same enzymes to obtain the final construct. This construct was designated as 33NHisTrxV3. Similar methodology was used to clone the V3 loop between residues 74 and 75 and between residues 83 and 84 of Trx.

Additional disulfides to constrain the inserted V3 peptides were designed. Since the protein 33NHisTrxV3 could not be purified and 83NHisTrxV3 did not generate antibodies against the V3 region (see below), the additional introduced disulfides were put only into 73NHisTrxV3. Two double mutants I307C/Y318C [hereafter referred to as 74NHisTrx V3(307)] and H308C/F317C [hereafter referred to as 74NHisTrxV3(308)] were constructed using megaprimer-based mutagenesis [41]. The whole gene was sequenced to confirm the mutations.

Expression of NHisTrx and its derivatives

Plasmids were transformed in *E. coli* strain DH5 α and grown in LB (Luria–Bertani) supplemented with ampicillin (100 μ g/ml). The cultures were grown to an D_{600} of 0.8, induced with 0.2% of arabinose and grown for 8 h at 37°C. For some constructs, cells were grown at 30 or 10°C after induction to increase the protein concentration in the soluble fraction of the cell lysate. Cells were lysed by sonication for 10 min. Protein was purified from the soluble fraction by affinity purification over a Ni-NTA (Ni²⁺-nitrilotriacetate) column (Qiagen, Germany). The yields of NHisTrx, 74NHisTrxV3, 74NHisTrxV3(307) and 74NHisTrxV3(308) were approx. 5 mg/l. 83NHisTrxV3 and 33NHisTrxV3 were absent from the soluble fraction and had to be purified from the insoluble fraction. After growth and cell lysis, inclusion bodies were resuspended in 6 M GdnCl in PBS for 8 h at room temperature (25°C). The solution was centrifuged at 9000 g for 30 min. Supernatant was bound on Ni-NTA beads and eluted with 6 M GdnCl in PBS containing 300 mM imidazole, at room temperature. The eluted sample was extensively dialysed against PBS at 4°C. Supernatant was collected and analysed by SDS/PAGE. 83NHisTrxV3 was purified with a yield of 2 mg/l, whereas 33NHisTrxV3 reprecipitated after dialysis and could not be further characterized.

Far-UV CD spectroscopy

CD spectra were recorded on a Jasco J-715 C spectropolarimeter (Jasco, Japan) flushed with nitrogen gas. The spectra were recorded in a 0.1 cm path length cuvette at a scan rate of 10 nm/min and a time constant of 8 s. All the data are an average over a minimum of six scans. Mean residue ellipticity was calculated as described previously [42].

SPR (surface plasmon resonance) studies

Binding affinities of TrxV3 constructs to various antibodies were determined by SPR using a Biacore2000 (Biacore, Uppsala, Sweden) optical biosensor at 25°C. In the binding assay, 500 RU (response units) of the full-length human mAb 447-52D

was attached by amine coupling with the surface of a research grade CM5 chip. Binding of gp120 and the TrxV3 derivatives to this surface was examined. A naked sensor surface without the antibody served as a negative control for the binding interaction. Serial dilutions of TrxV3 derivatives and gp120 were analysed at concentrations ranging from 50 to 500 nM in a buffer of PBS containing 0.05 % Tween 20. Binding and dissociation were measured for 75 and 300 s respectively at a flow rate of 30 μ l/min. In all cases, the sensor surface was regenerated between each binding reaction by using 1–3 washes of 10 mM NaOH for 60 s at a flow rate of 10 μ l/min. Each binding curve was corrected for non-specific binding by subtraction of the signal obtained from the negative control flow cell. Kinetic parameters were obtained by fitting the data to a simple 1:1 Langmuir interaction model by using BIAevaluation 3.1 software.

Binding data were analysed for at least four concentrations of each analyte. For competition studies, 447-52D was immobilized on a CM5 surface as described above and binding to gp120 was competed out by pre-incubating gp120 with different dilutions of immune sera before passing over the surface. As a control, gp120 pre-incubated with various known amounts of 447-52D was also passed over the surface. The concentration of 447-52D where the binding to the immobilized 447-52D was half that of free gp120 was designated as the IC₅₀ for 447-52D. The dilutions of sera where the binding of gp120 was half that of free gp120 was compared with the IC₅₀ value of 447-52D to determine an equivalent concentration of 447-52D-like antibodies in the immune sera.

Animal immunizations

All immunization experiments were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Guinea-pigs were injected intramuscularly with 40 μ g of protein formulated in PBS and containing 40 μ g of QS21 adjuvant (Antigenics, Lexington, MA, U.S.A). Animals were boosted with the same formulation at 4 and 8 weeks after the initial immunization. Sera were collected at 3, 8 and 12 weeks after the first injection.

Determination of antibody titres against the V3 loop and NHisTrx and viral neutralization assay

To determine the titre of antibodies directed against the V3 loop in the guinea-pig antisera, biotinylated-V3 loop peptide was immobilized on the streptavidin-coated surface of microtitre plates, at a concentration of 4 μ g/ml. After 3 washes, wells were blocked with 5 % (w/v) non-fat dried milk powder in PBST (PBS containing 0.1 % Tween 20). Wells were washed three times with PBST, and 70 μ l of serial dilutions of the serum were added. Incubation was carried out for 2 h at room temperature. Bound antibodies were detected with peroxidase-conjugated goat anti-guinea-pig antibody as described previously [40]. The reciprocal of the serum dilution showing an optical activity reading more than twice that of the negative control (pre-immune serum) and greater than 0.1 was taken as the antibody titre. To determine the titre against NHisTrx, microtitre wells were coated with NHisTrx (2 μ g/ml) overnight at 4 °C and a similar procedure was followed. All neutralization experiments were performed using a spread-based PBMC (peripheral blood mononuclear cells) assay [40] as described previously.

Determination of the total antibody concentration in serum

Guinea-pig immunosera (100 μ l) were bound at room temperature for 2 h to 100 μ l of Protein A–Sepharose (Amersham Biosciences, Piscataway, NJ, U.S.A). Beads were washed with PBS and eluted

with 5 mM glycine/HCl (pH 2.5). The pH was adjusted to 8.0 with 100 mM Tris/HCl (pH 8.0). The total antibody concentration was determined using a commercial BCA (bicinchoninic acid) assay (Sigma, St. Louis, MO, U.S.A) using BSA as the calibrating protein.

ELISAs to examine 447-52D binding to TrxV3 derivatives

To assess the binding of TrxV3 derivatives to 447-52D, 70 μ l of TrxV3 derivatives (5 μ g/ml) was immobilized in microtitre wells overnight at 4 °C. Wells were blocked as described above, washed three times and 70 μ l of different concentrations of 447-52D were added and wells were incubated for 2 h at 25 °C. Wells were washed again, and 70 μ l of a peroxidase-conjugated anti-human antibody was added per well and detected using *o*-phenylenediamine dihydrochloride. In this case, a well coated with gp120 was used as the positive control and NHisTrx-coated wells were used as negative controls.

Competition ELISA with peptides to map the epitope recognized by antisera

A set of five 15-residue peptides containing 11-residue overlaps and spanning the whole of the V3 sequence used for TrxV3 constructs was obtained from the NIH (National Institutes of Health) AIDS reagent programme (catalogue numbers 8837–8841) and used to compete out binding of either 447-52D or antisera to gp120. Briefly, gp120 was captured on D7324 coated wells as described previously [40] and incubated with either 60 μ l of 447-52D (5 μ g/ml) or 60 μ l of 10-fold diluted antisera that had both been pre-incubated with 2 μ g of the different peptides for 2 h at room temperature. Bound 447-52D or guinea-pig antibody was detected by peroxidase-conjugated anti-human antibody or peroxidase-conjugated anti-guinea-pig antibody respectively as described above.

RESULTS AND DISCUSSION

Design, construction, expression and purification of TrxV3 derivatives

Trx was used as a template for the insertion of V3 loop sequences. Trx was cloned into the *E. coli* expression vector pBAD24 to generate the NHisTrx construct with an N-terminal His tag. The insertions were designed based on the crystal structure of Trx (PDB code: 2trx) [43] and from the crystal structures of the V3 loop in complex with 447-52D (PDB code: 1qlj) [31]. The V3 loop complexed with 447-52D was chosen as the target structure, since designing an immunogen capable of eliciting a 447-52D-like antibody response was the goal of these studies. In order to present the V3 loop in a β -hairpin conformation similar to that adopted in the complex with 447-52D (Figure 1A), the V3 peptide was inserted in various loop regions of Trx. Trx was chosen as a scaffold as it is known to be a highly stable cytoplasmic soluble protein that can be expressed at high levels. We investigated insertion of the peptide at three different locations: between (i) residues 33 and 34, (ii) residues 74 and 76 and (iii) residues 83 and 85. Residues 74 and 76 as well as residues 83 and 85 occur in exposed loops of Trx [43]. There are previous reports in which various peptide coding sequences were inserted in the RsrII restriction site of the Trx gene (between residues 33 and 34) [37]. This position has also been used for display of random peptides [37,38]. The site Arg⁷³ in Trx is also a site that tolerates cleavage without causing unfolding of the protein and hence should tolerate insertion of a foreign peptide [44,45]. NHisTrx and 74NHisTrxV3 were purified from the soluble fraction of the cell lysate, while 83NHisTrxV3 was purified

Figure 1 Structure of the V3 peptide, Trx and the sequence of the V3 loop of JRFL gp120

(A) The conformation of the V3 peptide, derived from the MN strain of HIV-1, in complex with the 447-52D antibody [31]. Only the structure of the peptide is shown for clarity. (B) Structure of Trx [43] and the positions where the V3 peptide was inserted. The loop regions used for V3 peptide insertion are indicated with arrows. (C) The amino acid sequence of the V3 loop of JRFL gp120 is shown. Residues 305–320 comprising the V3 peptide insertion described in the text are highlighted in boldface. (D) Sequence alignment of residues 305–320 of the V3 peptide from HIV-1 strains JRFL, MN and IIB.

from inclusion bodies and refolded by dialysis. The latter protein was prone to precipitation upon storage. 33NHsTrxV3 was purified from inclusion bodies but precipitated upon refolding. Different refolding conditions were screened in a 96-well format as described previously [46], but no conditions to prevent the precipitation could be identified. Due to this problem, further biophysical and immunological characterization of this protein was not pursued.

In order to further constrain the conformation of the V3 loop in 74NHsTrxV3, disulfides were introduced at two different locations. The program MODIP [47] predicted that a possible disulfide could be formed between cysteine mutants of residues His³⁰⁸ and Phe³¹⁷. These residues formed a hydrogen-bonded pair between antiparallel β -strands. Our previous observations with naturally occurring disulfides between antiparallel β -strands suggested that such disulfides typically occur between non-hydrogen-bonded pairs [48]. Hence we also made a double mutant with a cysteine residue introduced at positions 307 and 318, a pair that was non-hydrogen-bonded. Both proteins were expressed and purified with yields similar to 74NHsTrxV3. Disulfide formation in both cases was confirmed by assaying for free thiols with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] and MS [48].

Characterization of the expressed proteins by CD spectroscopy

Far-UV CD spectra of NHsTrx, 74NHsTrxV3, 83NHsTrxV3, 74NHsTrxV3(307) and 74NHsTrxV3(308) are shown in Figure 2. Spectra for the four V3 loop derivatives of NHsTrx are similar to the spectrum for NHsTrx as well as the published spectrum for *E. coli* Trx [49]. This implies that the secondary structure and overall fold of NHsTrx are not appreciably disrupted by the insertion of the V3 loop in any of the cases.

Characterization of 447-52D binding to Trx derivatives by ELISA and SPR

Binding of 447-52D to immobilized TrxV3 derivatives was examined (Figure 3) by ELISA. 74NHsTrxV3, 83NHsTrxV3 and

Figure 2 CD spectra of Trx derivatives

Spectra for NHsTrx (1), 74NHsTrxV3 (2), 83NHsTrxV3 (3), 74NHsTrxV3(307) (4) and 74NHsTrxV3(308) (5) show that there is no significant structural change in Trx after the insertion of the V3 peptide

Figure 3 Direct binding of 447-52D to TrxV3 derivatives as assayed by ELISA

TrxV3 derivatives were immobilized on the microwell surface and incubated with various concentrations of 447-52D. The binding of 74NHsTrxV3(308) to 447-52D is significantly weaker compared with other TrxV3 derivatives. OD, absorbance.

74NHsTrxV3(307) bound 447-52D to a comparable extent, whereas the binding of 74NHsTrxV3(308) was weaker. The negative control in this case was wells immobilized with NHsTrx, which did not show any binding above background. This suggests that the V3 loop inserted in Trx was exposed and retained the ability to bind to 447-52D. Purified 74NHsTrxV3 and 83NHsTrxV3 proteins were further characterized using SPR to measure their binding affinity to immobilized 447-52D. The results are presented in Figure 4. The concentration-dependent binding of the positive control gp120 is shown in Figure 4(A), while Figures 4(B)–4(E) show results for constructs 74NHsTrxV3, 83NHsTrxV3, 74NHsTrxV3(307) and 74NHsTrxV3(308) respectively. NHsTrx was tested as a negative control (results not shown). The constructs lacking engineered disulfides and 74NHsTrxV3(307) show comparable binding to surface-immobilized 447-52D. Unlike the other constructs, binding data for 74NHsTrxV3(308) did not fit well to a simple 1:1 binding model.

Figure 4 SPR determination of kinetic and equilibrium dissociation constants for binding of gp120 and TrxV3 derivatives to immobilized 447-52D

Surface density, 1000 RU; buffer, 10 mM phosphate (pH 7.4), 150 mM NaCl and 0.0005 % Tween 20; flow rate, 30 μ L/min. Sensorgram overlays are shown for the binding of gp120 (A), 74NHsTrxV3 (B), 83NHsTrxV3 (C), 74NHsTrxV3(307) (D) and 74NHsTrxV3(308) (E). The analyte concentrations are indicated along with the curves. Data points are shown as filled circles and the fits as solid lines. No fits are shown for (E) as the data could not be reliably fitted. Only every fifth data point is shown for the curves (A–D) for convenience in visualization of the data.

Binding showed multiple phases (Figure 4E) and hence it was not possible to estimate a K_d value. However, the lower final RU (after 100 s) and rapid dissociation kinetics clearly indicate that this construct binds much more weakly to 447-52D than the other TrxV3 derivatives. Studies were carried out as a function of protein concentration to determine the rate constants for association and dissociation (k_{on} and k_{off} respectively) and the K_d value (k_{off}/k_{on}) for binding of the various constructs to 447-52D (Table 1). The kinetic parameters of binding were obtained using BIAevaluation 3.1 software. The binding parameters derived for 74NHsTrxV3, 74NHsTrxV3(307) and 83NHsTrxV3 binding to 447-52D were similar to the one obtained for gp120. The K_d

Table 1 Kinetic binding parameters for gp120 or TrxV3 derivatives to immobilized 447-52D as determined by SPR

Analyte	k_{on} ($\times 10^{-5}$ M $^{-1}$ s $^{-1}$)	k_{off} ($\times 10^3$ s $^{-1}$)	K_d (nM)
gp120	4	9	22.5
74NHsTrxV3	1.7	10	58
83NHsTrxV3	0.7	8	115
74NHsTrxV3(307)	2	8	40

could not be reliably estimated for 74NHsTrxV3(308), although this construct exhibited a much lower affinity than the rest as discussed above. This is either due to the importance of His³⁰⁸ in 447-52D binding or due to an altered conformation of the V3 loop due to the presence of a disulfide at a hydrogen-bonded pair. The similar binding affinities of gp120 and several of the TrxV3 derivatives for 447-52D suggest that the conformation of the V3 loop is similar in all cases. The K_d obtained for gp120 binding to 447-52D was also consistent with previous reports [50].

Immunogenicity and neutralization studies

Immunization of guinea-pigs with the constructs 74NHsTrxV3, 83NHsTrxV3, 74NHsTrxV3(307) and 74NHsTrxV3(308) was carried out. Table 2 shows that specific ELISA titres for all the antigens increased progressively from injection 1 to 3. It was found that 74NHsTrxV3 elicited a moderate V3-specific response, 83NHsTrxV3 and 74NHsTrxV3(307) exhibited significantly lower V3-specific titres and V3-specific titres in 74NHsTrxV3(308)-immunized animals were not detectable. Trx-specific antibodies were elicited to the same degree in animals immunized with 74NHsTrxV3 and 83NHsTrxV3, ruling out intrinsic inter-group differences. The low V3-specific titres elicited by 83NHsTrxV3 were most probably a result of the aggregation-prone nature of this protein. Trx-specific titres for 74NHsTrxV3(307) and 74NHsTrxV3(308) were lower than in other groups, suggesting that these immunogens may be aggregating or degrading *in vivo*. The low V3 titres seen in 74NHsTrxV3(308)-immunized animals also correlate well with the much weaker affinity of this protein for 447-52D. The mean titres of 74NHsTrxV3 antisera directed against the V3 loop peptide and native gp120 were comparable (20 800 versus 25 600), indicating that the V3 loop on JRFL gp120 is accessible for antibody binding. Table 3 shows ELISA and serum neutralization titres (IC₅₀) obtained after the third immunization. Neutralization studies were carried out on sera from all the animals receiving each antigen. The data show that neutralization activity against the neutralization-sensitive MN strain was present only in the case of 74NHsTrxV3, whereas no neutralization of JRFL or BAL strains could be observed. All three strains have very similar 447-52D epitope sequences and differences in their neutralization sensitivity to 447-52D and antisera are likely to be due to differences in V3 loop accessibility in the three strains. While the V3 loop is quite accessible to antibodies on monomeric JRFL gp120 as discussed above, it is likely that it is much less accessible in JRFL viral particles. This would explain the high IC₅₀ value (1.2 mg/ml) of 447-52D for JRFL (Table 3) seen in the neutralization. As expected, 83NHsTrxV3 antisera did not show neutralization activity against any of the strains. Hence, 83NHsTrxV3 antisera were not subjected to further characterization. Animals immunized with 74NHsTrxV3(307) and 74NHsTrxV3(308) also had low V3-specific titres and hence no further studies were carried out with these antisera.

Table 2 Trx- and V3-specific titres in guinea-pigs dosed with TrxV3 constructs

Bleeds were obtained at the indicated times following the first injection. Titres were determined using the method described in the Materials and methods section.

Immunogen	Serum number	Titre					
		3 weeks		8 weeks		12 weeks	
		Trx	V3	Trx	V3	Trx	V3
74NHISTrxV3	Serum 1	6400	1600	409600	6400	1638400	25600
	Serum 2	102400	1600	409600	6400	6553600	25600
	Serum 3	25600	6400	409600	6400	1638400	6400
	Serum 4	409600	6400	409600	25600	6553600	25600
83NHISTrxV3	Serum 5	25600	100	409600	100	1638400	100
	Serum 6	6400	400	409600	400	6553600	400
	Serum 7	400	< 100	102400	100	1638400	1600
	Serum 8	1600	100	409600	400	6553600	400
74NHISTrxV3(307)	Serum 9	< 100	< 100	1600	< 100	25600	6400
	Serum 10	< 100	< 100	< 100	< 100	6400	400
	Serum 11	< 100	< 100	6400	6400	102400	6400
	Serum 12	< 100	< 100	25600	< 100	6400	400
74NHISTrxV3(308)	Serum 13	< 100	< 100	6400	< 100	102400	< 100
	Serum 14	< 100	< 100	6400	< 100	102400	< 100
	Serum 15	< 100	< 100	25600	1600	25600	< 100
	Serum 16	< 100	< 100	400	< 100	25600	< 100

Table 3 Serum neutralization titres for guinea-pigs immunized with TrxV3 constructs as well as neutralization titres of mAb 447-52D against strains of primary isolates

Immunogen	Serum or antibody	Antibody titre after the third immunization		Neutralization titres (IC ₉₀)*						Approx. concentration of 447-52D-type antibodies (μg/ml)§
		Trx	V3	BAL		JRFL		MN		
				Pre†	PD3‡	Pre†	PD3‡	Pre†	PD3‡	
74NHisTrxV3	Serum 1	1638400	25600	<10	<10	<10	<10	<10	20	100
	Serum 2	6553600	25600	<10	<10	<10	<10	<10	40	400
	Serum 3	1638400	6400	<10	<10	<10	<10	<10	<10	ND
	Serum 4	6553600	25600	<10	<10	<10	<10	<10	10	50
83NHisTrxV3	Serum 5	1638400	100	<10	<10	<10	<10	<10	<10	ND
	Serum 6	6553600	400	<10	<10	<10	<10	<10	<10	ND
	Serum 7	1638400	1600	<10	<10	<10	<10	<10	<10	ND
	Serum 8	6553600	400	<10	<10	<10	<10	<10	<10	ND
447-52D		—	—	—	—	—	1.2 mg/ml	—	<9 μg/ml	

* IC₉₀ is 90% inhibitory concentration, expressed as the reciprocal of the serum dilution in which 90% or more of the viral antigen production was inhibited relative to production in untreated viral growth control wells. In the case of the antibody 447-52D, the antibody concentration at which 90% inhibition is observed is listed instead.

† Negative control with pre-immunization serum.

‡ Sera collected after the third injection (12 weeks after the first injection).

§ Concentrations were estimated using SPR (Figure 5) as described in the text.

|| ND, did not compete out 447-52D binding to gp120 even at dilutions of 50 μl/ml of serum.

Competition binding assays

The ability of guinea-pig immunesera from animals inoculated with 74NHISTrxV3 to compete with gp120 for binding to immobilized 447-52D was assessed as follows. Soluble gp120 (5 nM) was passed over a SPR chip containing immobilized 447-52D, either alone or after pre-incubation with various dilutions of sera. The total antibody concentration in the sera was approx. 8 mg/ml. As a positive control, a titration of free 447-52D incubated with gp120 was included. Pre-incubation with either sera or soluble 447-52D reduced the binding of gp120 to the surface. The dilution of the sera or the concentration of 447-52D where the binding was approximately half that of maximal is reported as the IC₅₀ (Figure 5). The IC₅₀ for soluble 447-52D was 200 ng/ml. The dilutions of the sera where 50% binding was observed were 2 μl/ml (serum 1), 0.5 μl/ml (serum 2) and 4 μl/ml (serum 3). The

comparison of the IC₅₀ values shows that the undiluted serum 1, serum 2 and serum 3 have 100, 400 and 50 μg/ml of 447-52D-like antibodies respectively. This is based on the assumption that antibodies present in the sera that bind to V3 do so with similar affinity as 447-52D. The negative controls (serum 5 and pre-immune serum) did not show any competition even at a dilution of 50 μl/ml. Descriptions of all sera are shown in Table 2. The amount of 447-52D-competitive antibodies present in each of the sera also correlated well with the neutralization titres for the sera. For example, the measured neutralization titre of serum 1 (PD3) for HIV-1 strain MN (Table 3) is 20. A 20-fold dilution of serum 1 would contain 5 μg/ml of 447-52D-like antibodies, consistent with the measured IC₉₀ of < 9 μg/ml for 447-52D. The most potent neutralizing serum had the highest concentration of 447-52D-like antibodies, whereas the least potent one had the lowest.

Figure 5 SPR-based competition of 447-52D with antibodies from sera obtained from animals immunized with 74NHisTrxV3

Sera were used at the indicated dilutions. gp120 alone (1) or pre-incubated with dilutions of 2 µl/ml of serum 1 (2), 0.5 µl/ml of serum 2 (3), 4 µl/ml of serum 3 (4) or 200 ng/ml of 447-52D (5) was passed over the 447-52D surface. The sensorgrams were obtained for several different concentrations of sera and 447-52D. However, only traces corresponding to 50% of the maximal binding in absence of antibodies are shown.

Epitope mapping of polyclonal antisera

A series of 15-mer peptides containing 11-residue overlaps was used to compete out gp120 binding to antisera or 447-52D. Figure 6 shows that only two peptides were capable of competing out binding of 447-52D and anti-74NHisTrxV3 antisera. One peptide contained the complete sequence used in the present study, while the other contained the first 11 residues of the sequence. The sequence of the V3-containing region in the latter peptide (KSIHIGPGRAF) matched with previous epitope mapping studies performed on 447-52D and also correlated with the previously reported crystal structure [31]. Neither Pep1 (TRPNNNT-**RKSIHIGP**, which lacks the terminal G of the GPG tip) nor Pep4 (**IGPGRAFYTT**GEIIG, which lacks the N-terminal region from 305–308) exhibited any competition with 447-52D or the antisera (epitope regions in each peptide are underlined in bold). This indicated that a substantial portion of the KSIHIGPGRAF epitope was targeted by both 447-52D and the antisera. The V3 peptide that co-crystallized with 447-52D [31] consists of residues 305–320 of HIV-1 MN envelope protein flanked by an additional cysteine residue at both the N- and C-termini. Peptide residues 305–309 (KRIHI) form an extended β -strand followed by a type II β -turn from residues 312–315 (GPGR). The β -strand has extensive main chain interactions with the antibody CDR H3 and forms a three-stranded β -sheet in conjunction with CDR H3. Residues 317–320 do not appear to interact with the antibody and are disordered in the crystal structure. The competition binding data shown in Figure 6 are consistent with the suggestion that antibodies generated against 74NHisTrxV3 are primarily directed against the N-terminal and GPGR regions of the V3 loop rather than the C-terminal region of the loop.

Thus, although 74NHisTrxV3 has the ability to induce a 447-52D-like response upon vaccination, the neutralizing capability of the immuneserum is poor. This is despite the fact that there is a reasonably high concentration of these antibodies, which we estimate at approx. 50–400 µg/ml of serum. In our assays, the neutralization titre for 447-52D against HIV-1 JRFL was 1.2 mg/ml (Table 3). Hence, the 447-52D mAb is itself only

Figure 6 Competition ELISA to map the epitopes of 447-52D and V3-specific antibodies in 74NHisTrxV3-elicited immuneserum

gp120 was coated on the microwell surface and the ability of a series of overlapping peptides, spanning the V3 peptide sequence used in the present study, to compete out the binding of (A) 447-52D or (B) anti-74NHisTrxV3 serum to gp120 was monitored. Peptide sequences correspond to HIV-1 consensus subtype B envelope amino acids 297–313 (Pep1), 301–317 (Pep2), 305–321 (Pep3), 309–325 (Pep4) and 313–328 (Pep5). The region of the V3 loop present in the TrxV3 constructs consisted of amino acids 305–320 of gp120. The conserved GPG tip consists of amino acids 312–314. Amino acids are numbered according to the reference HXB2 sequence [53]. OD, absorbance.

weakly neutralizing for neutralization-resistant strains such as JRFL. The neutralization titres reported here were consistent with a previously reported IC_{90} value of > 50 µg/ml measured using a single round of a pseudovirus neutralization assay [1]. It was reported in this recent study that 447-52D could neutralize up to 45% of clade B viruses at an IC_{50} of < 50 µg/ml but only 14% at an IC_{90} of < 50 µg/ml. Similar results were obtained for another antibody 58.2, which is directed against the HIGPGRAF V3 loop motif [51]. These data are also consistent with earlier studies [52] that showed that a number of primary isolates that contained the 447-52D epitope were poorly neutralized by this antibody and that neutralization was not predicted by antibody binding affinity to monomeric gp120. The data suggest that although 447-52D-like antibodies can be generated at reasonable concentrations in a polyclonal response, these antibodies are not able to effectively neutralize many primary viral isolates. This is presumably because of the low accessibility of the V3 loop in such isolates. Since the TrxV3 constructs described here are competent to generate antibodies directed against the tip of the V3 loop, they can serve as useful starting points to design immunogens containing analogous V3 loop sequences of non-clade B viruses. The neutralization properties of antibodies directed against the conserved GPGQ sequence present in a number of non-clade B viruses have not been extensively studied so far.

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